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TRANSMISSION OF *EHRLICHIA CHAFFEENSIS* FROM LONE STAR TICKS (*AMBLYOMMA AMERICANUM*) TO WHITE-TAILED DEER (*ODOCOILEUS VIRGINIANUS*)

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ABSTRACT: *Amblyomma americanum* is an aggressive ixodid tick that has been implicated as a vector for several bacterial agents. Among these is *Ehrlichia chaffeensis*, which causes human monocytic (or monocytotropic) ehrlichiosis. In this study, experimental tick transmission of *E. chaffeensis* from infected lone star ticks to deer was revisited, and the question of whether it would be possible to re-isolate the organism from deer was asked, because this had not been done previously. Here, we were able to transmit a wild strain of *E. chaffeensis* from acquisition-fed lone star ticks to white-tailed deer. *Ehrlichia chaffeensis* was re-isolated from one white-tailed deer on multiple days during the infection and from another deer on one day during the infection. Peak rickettsemias for *E. chaffeensis*-infected deer were 17 DPI with acquisition-fed ticks and 14 DPI with needle-inoculated deer. This study supports the role of the lone star tick and white-tailed deer as vector and reservoir host for *E. chaffeensis,* demonstrating culture re-isolation of *E. chaffeensis* in deer infected by experimental tick transmission for the first time.

Key words: *Amblyomma americanum*, deer, *Ehrlichia chaffeensis*, lone star ticks, tick-borne diseases, transmission.

INTRODUCTION

Ehrlichia chaffeensis is one of a number of tick-borne agents transmissible by lone star ticks (LST; *Amblyomma americanum*). It is an obligate intracellular rickettsial agent that invades and replicates in mononuclear cells and causes monocytic (or monocytotropic) ehrlichiosis in humans. Humans who are more severely infected are often immunocompromised in some way, such as being infected with HIV, elderly, or a transplant recipient. Symptoms are typically flu-like and usually self-limiting in healthy individuals.

The natural transmission cycle for *E. chaffeensis* involves LST as the vector and white-tailed deer (*Odocoileus virginianus*) as the reservoir host (Lockhart et al., 1997a). White-tailed deer show no clinical signs of infection and can be persistently infected, making them ideal reservoirs (Davidson et al., 2001). In addition to deer, other animals can be infected with *E. chaffeensis*. For example, dogs have been experimentally infected with the agent, and natural infections in dogs have also been detected by serologic and molecular testing, making this agent a veterinary as well as medical concern (Dawson and Ewing, 1992; Dawson et al., 1996; Breitschwerdt et al., 1998; Murphy et al., 1998; Pretorius and Kelly, 1998; Zhang et al., 2003). *Ehrlichia chaffeensis*, or antibodies reactive against the organism, have been detected in naturally exposed lemurs (*Lemur catta*, *Eulemur macaco flavifrons*, and *Varecia variegate variegate*) (Williams et al., 2002; Yabsley et al., 2004), coyotes (*Canis latrans*) (Kocan et al., 2000), raccoons (*Procyon lotor*), and opossums (*Didelphis virginianus*) (Lockhart et al., 1997b). Red foxes (*Vulpes vulpes*) have been experimentally infected with *E. chaffeensis* (Davidson et al., 1999). All, and possibly others, might be involved in the epidemiology of the organism.

Ewing et al. (1995) demonstrated transmission of *E. chaffeensis* from nymphal and adult LST to deer based on molecular and serologic detection of infection. At that time, they were unable to re-isolate *E. chaffeensis* from deer. In an attempt to

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confirm that *E. chaffeensis* can be transmitted from LST to deer, we repeated the experiment using a wild strain of *E. chaffeensis* and attempted transmission through acquisition feeding of adult LST on three white-tailed deer fawns. In this study, infection of deer was confirmed by positive culture. This supports and complements the study by Ewing et al. (1995), lending more evidence to the role of white-tailed deer as a reservoir host for *E. chaffeensis.*

MATERIALS AND METHODS

Acquisition-fed ticks

In summer of 2004, 11 white-tailed deer fawns were reared in tick-free facilities as previously described (Varela-Stokes et al., 2006). All deer tested negative for *E. chaffeensis, Ehrlichia ewingii, Anaplasma* of white-tailed deer, *Anaplasma phagocytophilum, Bartonella* spp., and *Borellia lonestari* by polymerase chain reaction (PCR), and were negative for antibodies to *E. chaffeensis, A. phagocytophilum,* and *B. lonestari.* Animals were handled within approved guidelines set forth by the Institutional Animal Care and Use Committee (AUP# A2005-10251-c1). Deer were weaned at 3 mo of age and five were subsequently inoculated with approximately 2.4×10^6 DH82 cells (a continuous canine macrophage cell line) infected with a wild strain of *E. chaffeensis* (HH604-2 from Greene County, Arkansas; 36°04′28″N, 90°22′37″W). This strain has five repeats, approximately 90 base pairs in length, in the variable length PCR target (VLPT) gene.

Blood was collected from deer three times weekly for 28 days for PCR, serology, and isolation in tissue culture. On day postinfection (DPI) 7, approximately 1,500 nymphal LST in feeding chambers were attached to the backs of two of the five inoculated deer (numbers 480 and 481); chambers remained on the deer until DPI 12, at which point ticks had fed to repletion. Ticks were removed and maintained at 94% humidity where they molted to adults and remained for approximately 9 mo. Engorged nymphs, nymphs in the process of ecdysis, and unfed adult ticks were tested by PCR to verify presence of organism and transstadial transmission. Blood samples from the five infected deer were used to determine the peak level of rickettsemia by real-time PCR, described below.

Transmission feeding

Three white-tailed deer fawns were reared in tick-free facilities in the summer of 2005. Deer were PCR and antibody negative for the same organisms as were tested in deer the previous year. At approximately 3 mo of age, deer were weaned and were entered into the study. We used adult LST that had acquisition-fed as nymphs on the two infected deer the previous year. Approximately 300 ticks were placed in feeding chambers attached to the backs of the three deer. Uninfected nymphs (less than 50) were also placed in chambers to test whether nymphs would become infected by cofeeding. Two deer (numbers 19 and 25) had approximately 150 male and 150 female ticks that had previously fed on one inoculated deer (number 481) the previous year; the remaining deer (number 24) had a mixed population of approximately 100 male and 200 female ticks that fed on two inoculated deer (numbers 480 and 481) the previous year. Chambers were removed 10 days after initial placement because of problems with detachment. Remaining ticks were removed for PCR testing with the male ticks pooled in groups of five or six; due to engorgement, female ticks were tested individually. Blood was collected three times weekly to monitor infection by PCR, serology, and isolation. One negative control deer (housed in the same building) that did not receive ticks was PCR tested on DPI 14 and 28 to check for presence of *E. chaffeensis.*

PCR

For DNA extraction from whole blood, we used a GFX Genomic Blood DNA Purification Kit (GE Healthcare, Piscataway, New Jersey, USA). For ticks, the method of extraction varied. Engorged nymphs or engorged adult ticks were homogenized in 500 μl of extraction solution with the remainder of the GFX extraction followed according to published protocol. Unfed adult ticks and male-fed adults were dissected and tissues, including midgut and salivary glands, were placed in 130 μl phosphate buffered saline (PBS, pH 7.4). For the GFX extraction protocol, 50 μl of tissue homogenate or suspension were used.

Two PCR targets were used to test for the presence of *E. chaffeensis* in nested PCR assays. For the VLPT gene, primers FB5A and FB3A were used in the primary reaction, and FB5 and FB3 were used in the secondary reaction. For the 16S rRNA gene, primers ECC and ECB were used in the primary reaction, and HE1 and HE3 in the secondary

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reaction. Reaction conditions and primers have been previously described (Little and Howerth, 1999; Sumner et al., 1999; Varela et al., 2005). Products were electrophoresed on 2% agarose gels stained with ethidium bromide.

Real-time PCR

The LUX™ (Light Upon eXtension) system (Invitrogen, Carlsbad, California, USA) was used for real-time PCR. The fluorogenic reverse primer, labeled with FAM and the corresponding unlabeled forward primer were designed using the LUX™ Designer software. The sequences of both primers were Echaff 16S_272RL 5′ GACGATTTCCAGTGTGG CTGATCGTC 3′ and E chaff 16S_255F 5′ TGGCTTACCAAGGCTATGATCT 3′. The product was 64 bp long. Real-time PCR reactions were done in 25 µl reactions using 12.5 µl of 2× Platinum Quantitative PCR Supermix UDG (Invitrogen), 0.5 µl of each primer (final concentration 200 nM), 0.5 µl ROX reference dye, 6 µl distilled water, and 5 µl DNA template. The ROX reference dye was diluted 1:10 before adding to the master mix. DNA template was extracted from blood samples as previously described above. We used a Stratagene Mx3000P (Stratagene, La Jolla, California, USA) with reaction conditions as follows: 50 C for 2 min, 95 C for 2 min, followed by 40 cycles of 95 C for 15 sec, 60 C for 30 sec, and 72 C for 30 sec. This was followed by 95 C for 1 min, and melting curve analysis (55 C for 30 sec and 95 C for 30 sec with data acquired for all temperature points). We used the FAM channel to detect fluorescence. We tested all samples in duplicate and on at least two separate occasions.

For standards, we used a known concentration of *E. chaffeensis* DH82-infected cells (50 cells/µl) and made serial dilutions. The concentration of infected cells was determined by harvesting a flask of *E. chaffeensis-infected* DH82 cells, counting the total number of cells using a hemacytometer, and determining the percent of infected cells by staining a cytospin of the harvested cells. We used these as our standards because we felt they would more realistically reflect the number of infected cells in a sample, keeping in mind that infected DH82 cells would likely have more morulae than naturally-infected monocytes.

Serology

To determine antibody titers, we used an indirect fluorescent antibody test utilizing antigen from culture grown *E. chaffeensis* (Dawson et al., 1991). Sera from DPI 0, 3, 7, 12, 17, 21, 28, and 31 were screened at a concentration of 1:64 and positive samples were tested at serial two-fold dilutions. We used a 1:30 dilution of fluorescein isothiocyanate-labeled rabbit antideer (KPL, Gaithersburg, Maryland, USA) to detect *E. chaffeensis*.

Isolation in tissue culture

For isolation of *E. chaffeensis* in culture, 5–7 ml EDTA anticoagulated blood were aseptically collected from deer twice weekly and samples were prepared by lysing red blood cells and inoculating white blood cells, as previously described, onto a monolayer of DH82 cells (Varela et al., 2003). Cultures were maintained for 45 days or until evidence of cytopathic effect, at which point cultures were harvested, cytospins prepared and stained with Dif Quik to confirm results.

RESULTS

Based on PCR results for the 16S rRNA gene target, the prevalence of infected engorged nymphal LST that had acquisition-fed on two deer was 15% (3/20 LST), whereas prevalence in ticks in the process of undergoing ecdysis was 25% (5/20 LST). Of fifty unfed adult LST that had molted from engorged nymphs, 4% that fed on deer number 480 were PCR positive and 6% that fed on deer number 481 were PCR positive. PCR using the VLPT gene target confirmed that these ticks were infected with a five-repeat strain of *E. chaffeensis*; this is consistent with the *E. chaffeensis* strain that was used to inoculate the deer used for acquisition feeding.

The results of the experimental tick transmission are shown in Table 1. Briefly, one deer was PCR positive from DPI 12 to DPI 26, and culture positive from DPI 7 to DPI 28. We detected *E. chaffeensis* by PCR and culture in another deer on one day (DPI 14); there was no evidence of infection in the third deer throughout the trial. The negative control deer was PCR negative on both days tested. Only deer number 24 developed antibodies against *E. chaffeensis*, having a peak titer of 512

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TABLE 1. PCR, culture, and serologic results of white-tailed deer (WTD) from transmission of *E. chaffeensis* from acquisition-fed nymphs to white-tailed deer. PCR results in parentheses denote the number of VLPT repeats detected.

	WTD number	Days post-tick placement														
		0	3	5	7	10	12	14	17	19	21	24	26	28	31	33
PCR	19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	24	-	-	-	-	-	+ (5)	+ (5)	+ (5)	+(5)	+ (5)	+ (5)	+ (5)	-	-	-
	25	-	-	-	-	-	-	+ (5)	-	-	-	-	-	-	-	-
Culture	19	nd[a]	nd	-	-	-	nd	-	-	nd	-	-	nd	-	-	-
	24	nd	nd	-	+	+	nd	+	+	nd	+	+	nd	+	-	nd
	25	nd	nd	-	-	-	nd	+	-	nd	-	-	nd	-	-	nd
Serology	19	-	-	nd	-	nd	-	nd	-	nd	-	nd	nd	-	-	nd
	24	-	-	nd	-	nd	-	nd	1:128	nd	1:512	nd	nd	1:512	1:256	nd
	25	-	-	nd	-	nd	-	nd	-	nd	-	nd	nd	-	-	nd

[a] nd denotes "not done."

on DPI 21 and DPI 28. Overall, titers appeared lower than titers reported from deer inoculated via syringe with *E. chaffeensis* (Davidson et al., 2001; Varela et al., 2003), The number of VLPT repeats detected in both PCR-positive deer was five, which did not differ from that of the infected deer used for acquisition-feeding LST. Detection of *E. chaffeensis* in adult fed females from the deer that was PCR positive on multiple days was 10%; female ticks from the other two deer were PCR negative for *E. chaffeensis*. All pools of fed males were also PCR negative. One nymph recovered from one deer was PCR negative; the remaining nymphs were lost due to chambers falling off.

Using real-time PCR, we determined that the peak level of infection for the five needle-inoculated deer was DPI 14, at a level of 7.4 cells per 1 ml of blood, and peak for deer infected with E. chaffeensis by acquisition-fed nymphs was DPI 17 with a level of 0.024 infected cells per 1 ml of blood. Because the standards are likely an overestimate of the number of morulae within an infected deer monocyte, a more accurate interpretation of these results would be the total level of bacteria within any number of infected cells in 1 ml of blood.

DISCUSSION

Several characteristics of white-tailed deer make them ideal reservoir hosts for *E. chaffeensis.* First, they do not experience detectable morbidity or mortality from infection with the organism, and second, they can be persistently infected for up to 9 mo (Davidson et al., 2001). Third, deer are the preferred host for LST and are frequently infested with large numbers of these ticks (Kollars et al., 2000). In this study, the cultivation of *E. chaffeensis* from deer infected via tick transmission confirms that deer can be infected through feeding of infected LST. This supports previous PCR evidence for *E. chaffeensis* transmission via this vector (Ewing et al., 1995). The demonstration of viable organism in deer blood on multiple occasions after tick feeding not only verifies that that transmission was successful but demonstrates prolonged availability of *E. chaffeensis* to feeding LST. Furthermore, support for LST vector competence is provided by demonstration of transstadial transmission; in this study, engorged nymphs, molting nymphs, and adult ticks all showed molecular evidence of *E. chaffeensis.* Only one of three deer was positive on more than one sample day during the trial; however, organism was

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re-isolated consistently from this deer as well as the deer that was transiently positive on DPI 14.

Unfortunately, due to problems with tick chambers falling off during transmission feeding, ticks had to be removed two days earlier than was planned. Because many of the females were only partially engorged, this might have precluded the transmission of *E. chaffeensis* from infected ticks in the deer that did not become positive or the deer that was transiently positive. Despite this, it appears that there was minimal effect on the study because two of the three deer did show some evidence of infection. In this trial, real-time PCR demonstrated a peak in rickettsemia on DPI 17, more closely comparable to that of needle-inoculated deer. However, the level of organism detected was significantly lower, likely owing to the paucity of organism in ticks, as compared to a culture-derived inoculum. In addition, infected DH82 cells might have a larger number of morulae within individual cells; thus our standards might not have accurately reflected the amount of ehrlichiae within naturally infected monocytes and might be a better measure of the total level of bacteria in that sample of blood.

Interestingly, anti-*E. chaffeensis* antibodies were only detected in the one exposed deer that became positive by PCR and culture isolation. The deer that showed transient infection did not seroconvert; however, field data have shown that approximately 35% of naturally-infected deer that are PCR positive are also seronegative (Yabsley, M. J., pers. comm.). Thus, this is not unusual. The deer that did not show evidence of infection was also seronegative. It does not seem likely that none of the ticks that fed on deer harbored *E. chaffeensis* because organism was detected in 6% and 4% of tested adults prior to tick feeding. Still, this might have been possible and might attribute to the lack of seroconversion.

Amblyomma americanum transmits a number of agents known or suspected to cause disease. Because all three stages of LST prefer to feed on white-tailed deer, these vertebrates are ideal reservoir hosts. Apart from the study by Ewing et al. (1995), the transmission of *E. chaffeensis* from LST to white-tailed deer largely has been based on circumstantial evidence. Furthermore, although the Ewing et al. (1995) study intended to demonstrate transmission by LST, it lacked culture confirmation, being dependent on seroconversion and PCR detection. One study performed in 2001 attempted to feed LST on day 243 postinoculation of deer with *E. chaffeensis* (Davidson et al., 2001). Despite detection of *E. chaffeensis* DNA in one of the deer on day 278 postinoculation, ticks remained PCR negative. This could be explained by the fact that this deer was also PCR and culture negative during the time of tick feeding. This implies that the probability of LST becoming infected with *E. chaffeensis* by feeding on an infected white-tailed deer could vary over the course of that infection. Here, demonstrating re-isolation of organism from an infected deer further implicates LST as a suitable vector, supports white-tailed deer as a reservoir host, and improves our understanding of the natural history of this bacterial agent.

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